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SOME CONTRIBUTIONS OF PNEUMOCOCCAL GENETICS TO THE ELUCIDATION OF BIOLOGICAL PHENOMENA*

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ONE of the more interesting outgrowths of medical microbiology has been the utilization of pathogenic bacteria in studies designed to elucidate fundamental biological problems. Prior to the advent of chemotherapeutic and antibiotic agents, such organisms were the object of continuing scrutiny by many workers, and few among them were studied more intensively than was the pneumococcus. In his monograph published in 1936, White[1] included a bibliography of 1593 references related to this organism. Although little clinical interest in pneumococcus is manifested by physicians today, information accumulated previously from studies of pneumococcus has provided the basis for a continuing examination of its properties as a living cell. As a result thereof, infor-

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mation germane to several of the biological sciences is still being derived from investigations concerned with the genetics of this organism.

To begin, from the study of pneumococcus comes the most direct evidence concerning the chemical nature of genes, the determinants of inheritance. In 1928, the British bacteriologist, Griffith[2], reported his extraordinary findings relating to capsulation in pneumococcus. In studies designed to discover what conditions would favor alteration of avirulent noncapsulated pneumococci to the virulent capsulated state, Griffith employed several experimental techniques. Among them was the injection subcutaneously into mice of living noncapsulated organisms derived from capsular type II together with heat-killed capsulated type II cells. A number of the animals died and, from their blood, living virulent capsulated type II pneumococci were recovered. The experiment was repeated on another occasion, but with the substitution of heat-killed capsulated type I cells for the vaccine of pneumococcus type II employed initially. On this occasion, too, a number of the animals succumbed to pneumococcal infection, but the organism recovered at autopsy was not pneumococcus type II but type I! The result must have been a startling one, for a pneumococcus derived from a strain of one capsular type was found now to be producing the capsular polysaccharide of an organism of a different capsular type. The possibility that one or more viable type I pneumococci had survived in the "heat-killed" vaccine, accounting thereby for the anomalous results, concerned Griffith greatly and he repeated his experiments with scrupulous controls. The results were the same. His findings were confirmed by Neufeld and Levinthal[3]; and, shortly thereafter, a systematic study of the phenomenon was undertaken in the laboratory of Avery at the Rockefeller Institute, where investigations of the phenomenon of pneumococcal capsular type transformations were to continue over a period of nearly two decades. As a result thereof, Avery, MacLeod and McCarty[4] published in 1944 their important observation that the material extractable from capsulated pneumococcal cells which was capable of inducing the formation of a capsule by noncapsulated cells exposed to it was apparently deoxyribonucleic acid. For the first time, a specific biological activity could be ascribed to a chemical substance of this class. Another finding of note was that the deoxyribonucleate or "transforming principle" behaved within the cell as a self-replicating unit, for it could be recovered from the combined progeny

of the transformed cell in amounts far in excess of that required to induce the initial transformation. The attribution of genetic activity to deoxyribonucleate was consistent with observations on other than bacterial cells; for, in most cells, substances of this class are confined to the nucleus. Further studies of the chemistry of "transforming principle" by McCarty[5] and by Hotchkiss[6] have left little doubt that the conveyor of genetic information resides in deoxyribonucleate. Although it is known today that genetic information governing the structure of some viruses is contained in ribonucleate, no system permitting cellular or viral replication in the absence of deoxyribonucleate has yet been described.

The tentative structure of deoxyribonucleate proposed by Watson and Crick[7] appears suited in many ways for the incorporation and transmission of genetic data. They have propounded a model consisting of a two-stranded helix held together by the hydrogen bonds between complementary pairs of purine and pyrimidine bases. The sequence of bases is thought to determine the genetic information carried by the deoxyribonucleate molecule. If separation of the two strands of the helix can occur, two templates are provided thereby for the formation of new complementary strands of the same sequences as were present in those of the original molecule. Such a structure meets many of the requisites for genetic activity.

The demonstration of the chemical nature of the genetically active material in pneumococcus has provided, therefore, the first and most direct evidence of the biochemical nature of genes. If this inference is correct, then it should be possible to show that cellular characters other than the pneumococcal capsule are subject to hereditary control by deoxyribonucleates, and such has proved to be the case. The inheritance of a variety of cellular properties can now be modified in the transforming system in a fashion analogous to that demonstrable for capsular polysaccharide. Possession of a technique for controlling in a predictable fashion the inheritance of such cellular attributes has facilitated the collection of data pertinent to several biological disciplines, and it is with these data that the remainder of this report will be concerned.

By means of bacterial transformation reactions, it is possible to demonstrate the genetic basis of bacterial resistance to chemotherapeutic and antibiotic agents and to show that such resistance can be transferred

from resistant to sensitive cells by growing the latter in the presence of deoxyribonucleate of the former cells. In this fashion, resistance to sulfonamides, optochin, penicillin, erythromycin and streptomycin has been transferred from resistant to sensitive cells. Of additional interest is the fact that the mutational pathway to drug resistance may be reflected by the deoxyribonucleate of the resistant cell[8]. Resistance to high concentrations of streptomycin may arise in pneumococcus as the result of a single mutation or may be the result of multiple mutational steps. If cells of a streptomycin-sensitive strain of pneumococcus are exposed to the deoxyribonucleates of the one-step mutant, a high degree of resistance to this drug will be acquired by the cells transformed. A similar population of sensitive cells exposed to the deoxyribonucleates of the multistep mutant will acquire only a fraction of the resistance manifested by the donor of the genetic material, because the probability that a given cell will acquire more than one recognizable genetic factor in a single transformation is small. If re-exposed to the same preparation of deoxyribonucleates, however, additional increments of resistance may be acquired. In this way, the genetic history of the resistant strain is stored in its deoxyribonucleates. These observations provide direct confirmation of the genetic basis of drug resistance in bacteria. Their relevance to pharmacology and to clinical medicine is obvious.

It has been possible to show also by means of transformation reactions that the enzymatic activities of pneumococcus are subject to genetic control. The ability of cells to ferment salicin[9] and mannitol[10] can be modified by such reactions, as can certain of the enzymatic activities essential to the formation of the pneumococcal capsule[11]. Additional information of interest to biologists and to biochemists has been derived from a study of the adaptive enzyme, mannitol dehydrogenase, essential for the utilization by pneumococcus of the hexose alcohol, mannitol. An adaptive enzyme is one that is present in a cell in minimal quantities in the absence of its substrate, but which increases in amount after exposure to that substrate. The latter phenomenon is called induction. A cell which has been induced will utilize substrate immediately, whereas the uninduced cell will not do so until a finite time interval required for induction has elapsed. Marmur and Hotchkiss[10] have examined the deoxyribonucleates of uninduced and induced cells carrying the gene which determines the presence of the enzyme, man-

nitol dehydrogenase. When cells lacking this enzymatic activity are transformed with either of these deoxyribonucleates, they behave as uninduced cells; i.e., cells capable of forming the enzyme but failing to do so in the absence of exposure to the substrate. The experiment demonstrates that the deoxyribonucleate conveys only the ability to form the adaptive enzyme and that adaptation itself is a cellular function which is distinct from this genetic capability.

Transformation reactions have been useful in elucidating a variety of problems in the field of bacteriology. Although pneumococcus is known by the name *Diplococcus pneumoniae* in the United States, it is designated *Streptococcus pneumoniae* by British bacteriologists. Studies of several alpha hemolytic strains of streptococcus have shown them to be related genetically to pneumococcus. It has been possible to confer resistance to streptomycin upon a sensitive pneumococcus with the deoxyribonucleates of streptococci resistant to this drug and to carry out the genetic transfer of drug resistance in the opposite direction as well[12]. It is highly unlikely that these so-called "interspecific" transformations could be effected if there were not very close similarities in the deoxyribonucleates of the strains involved, and the results suggest an evolutionary relationship among the organisms concerned.

It is of interest that the efficiency of transfer of the genetic unit controlling resistance to streptomycin is usually lower in "interspecific" transformation than in "intraspecific" transformation. Interspecific transfer of the genetic determinants of more complex functions such as those controlling the formation of a bacterial capsule has not been accomplished to date.

The relation of bacterial colonial morphology to virulence has been a subject of interest both to bacteriologists and physicians. Although certain relationships appear to exist between these two attributes of bacteria, well-recognized discrepancies have disturbed what has appeared otherwise to be a useful correlation. Studies employing genetic recombination in pneumococcus have been helpful in clarifying this problem[13, 14]. It has been shown that the production of "rough" or "smooth" colonies is dependent primarily upon the mode of cellular separation after division, and that the presence or absence of the mucoid colonial state is dependent upon the amount of capsular polysaccharide produced by the cells comprising the colony. By the use of appropriate se-

lective cultural techniques and of genetic recombination in transformation reactions, it can be demonstrated in pneumococcus that mode of cellular separation after division and formation of a capsule are genetically distinct cellular properties. In addition, it can be shown in pneumococcus that virulence is dependent largely, if not entirely, upon the presence of the capsule and is independent of the mode of cellular separation after division. It has been found also that cells forming large amounts of capsular material give rise to mucoid colonies in which the pattern of cellular separation after division may be masked. The experiments show clearly the limitations of attempting to correlate colonial morphology with virulence and also the importance of examining bacterial cells rather than colonies in attempting to relate cellular properties to virulence.

Another finding arising from the genetic study of pneumococcus has been the recovery, following transformation, of strains which produce simultaneously two capsular polysaccharides[15]. These strains provide a new basis for serological cross-reactivity in this species and are therefore of immunological interest. Unlike strains which cross-react serologically by virtue of the fact that the molecules of their single dissimilar capsular polysaccharides contain common chemical subgroups, the strains with binary capsules produce two distinct capsular polysaccharides which are separable by a variety of techniques. That two distinct polysaccharides are produced can be demonstrated with precipitin reactions in an agar gel. These strains are of genetic as well as of immunologic interest, for they result from the simultaneous presence within the cell of the normal capsular genome of a strain which includes a uronic acid in its capsule together with the mutated capsular genome of a type III strain which has lost the ability to produce a capsule[11]. Loss of capsulation by the mutated type III strain results from loss of the ability to form glucuronic acid, an essential constituent of type III polysaccharide. When an appropriate heterologous capsular genome is introduced by transformation into the mutated type III cell without concomitant loss of the mutated type III capsular genome, the transformed cell is apparently able to divert some of the glucuronic acid formed under the influence of the newly introduced capsular genome to the production of type III polysaccharide; and both this capsular polysaccharide as well as that determined by the newly introduced capsular genome are expressed by the cell. The competition for glu-

curonic acid leads, however, to partial suppression of the polysaccharide determined by the newly introduced capsular genome as well as to augmented synthesis of type III capsular material. The phenomenon of binary capsulation provides, therefore, an example of genetic suppression and augmentation explicable in terms of a competitive biochemical reaction.

The epidemiological implications of genetic recombination are of considerable interest. It has been shown by Ephrussi-Taylor[16] that genetically distinct, noncapsulated variants of pneumococcus type III which are lacking in virulence may be recognized in transformation reactions, even though these organisms appear similar when examined by more conventional techniques. In such reactions, the deoxyribonucleate of one strain will interact with that in cells of the other strain in such a fashion as to lead to the restitution of the normal type III capsule. The relationship is a reciprocal one, either cell being capable of acting as the donor or the recipient of the deoxyribonucleate. In a reaction of this kind, the genetic units of two avirulent cell lines interact to give rise to a fully virulent pneumococcus. If recombinations of this kind were to occur under natural conditions, they might account for certain of the phenomena of epidemiology which evoked the doctrine of spontaneous generation in former times. In an experimental model such as the one described, two avirulent organisms interact to give rise to a virulent strain where none was present before. Such an event would provide circumstances which might account for the sudden appearance of a bacterium responsible for an explosive outbreak of infection. It is noteworthy that the capsule of meningococcus, an organism known for its epidemic potentialities, can also be altered hereditarily in transformation reactions[17].

Does bacterial transformation occur under natural conditions? Proof of such an event is lacking at the present time, though there exists circumstantial evidence compatible with an affirmative answer to this question. Pneumococcus produces a type-specific, somatic M protein which varies independently of type-specific capsular polysaccharide[18, 19]. Examination of numerous pneumococcal strains shows that different M proteins may be associated with organisms of the same capsular type, and that the same or closely similar M proteins may be found in pneumococci of different capsular types. One explanation for these antigenic relationships is that they may have arisen through genetic recombination,

for such recombinations can be accomplished in transformation reactions. Second, by employment of the technique of Griffith, it has been possible to demonstrate the occurrence of capsular transformation in a variety of mammalian species, including the rat, rabbit, guinea pig, cat and rhesus monkey[20]. In none of these species, however, has it been possible to demonstrate transformation of the pneumococcus in its usual habitat, the respiratory tract, be it normal or damaged by viral infection. It has been shown, *in vitro*, that transformation can be effected with deoxyribonucleate from a relatively small number of pneumococci[21]. Failure to demonstrate such genetic recombination in the respiratory tract may reflect ignorance of the conditions necessary for the occurrence of such a reaction rather than the inability of the reaction to take place. It should be pointed out also that spontaneous mutation in the direction of increased virulence may occur in pneumococcus and in other species of bacteria, and that it is not necessary to invoke genetic recombination as the sole means of accounting for the appearance of potentially epidemic strains of bacteria.

Since the discovery of the pneumococcal transformation reaction, other mechanisms of genetic recombination in bacteria have been recognized. A phenomenon akin to sexual recombination has been found to occur in the colon bacillus; and, in the salmonellas, part of the bacterial genome may be transported from one strain to another by bacterial virus known as bacteriophage. From the study of all these types of genetic recombination has come a wealth of material pertinent to a variety of biological problems. The essential similarity of living cells, the large numbers of bacterial cells which can be handled in the laboratory and their short generation time make bacteria suitable for the study of a wide diversity of biological phenomena. It may be anticipated that additional information of fundamental importance will be forthcoming from the continuation of such studies.

REFERENCES

1. White, B. *Biology of Pneumococcus*, New York, Commonwealth Fund, 1938.
2. Griffith, F. Significance of pneumococcal types, *J. Hyg. 27*:113-59, 1928.
3. Neufeld, F. and Levinthal, W. Beiträge zur Variabilität der Pneumokokken, *Z. ImmunForsch. 55*:324-40, 1928.
4. Avery, O. T., MacLeod, C. M. and McCarty, M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. I. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III, *J. exp. Med. 79*:137-58, 1944.
5. McCarty, M. and Avery, O. T. Studies

on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonuclease on the biological activity of the transforming substance, *J. exp. Med. 83*:89-96, 1946.

6. Hotchkiss, R. D. Études chimiques sur le facteur transformant du pneumocoque, *Colloq. int. Cent. nat. Rech. sci. 8*:57-65, 1949 (Unités Biologiques Douées de Continuité Génétique).
7. Watson, J. D. and Crick, F. H. C. Structure of DNA, *Cold Spr. Harb. Symp. quant. Biol. 18*:123-31, 1953.
8. Hotchkiss, R. D. Genetic chemistry of the pneumococcal transformations, *Harvey Lectures 49*:124-44, 1954.
9. Austrian, R. and Colowick, M. S. Modification of the fermentative activities of pneumococcus through transformation reactions, *Bull. Johns Hopk. Hosp. 92*: 375-84, 1953.
10. Marmur, J. and Hotchkiss, R. D. Mannitol metabolism, a transferable property of pneumococcus, *J. biol. Chem. 214*:383-96, 1955.
11. Austrian, R., Bernheimer, H. P., Smith, E. E. B. and Mills, G. T. Simultaneous production of two capsular polysaccharides by pneumococcus. II. Genetic and biochemical bases of binary capsulation, *J. exp. Med. 110*:585-602, 1959.
12. Bracco, R. M., Krauss, M. R., Roe, A. S. and MacLeod, C. M. Transformation reactions between pneumococcus and three strains of streptococci, *J. exp. Med. 106*:247-59, 1957.
13. Austrian, R. Morphologic variation in pneumococcus. I. An analysis of the bases for morphologic variation in pneumococcus and description of a hitherto undefined morphologic variant, *J. exp. Med. 98*:21-34, 1953.
14. Austrian, R. Morphologic variation in pneumococcus. II. Control of pneumococcal morphology through transformation reactions, *J. exp. Med. 98*:35-40, 1953.
15. Austrian, R. and Bernheimer, H. P. Simultaneous production of two capsular polysaccharides by pneumococcus. I. Properties of a pneumococcus manifesting binary capsulation, *J. exp. Med. 110*:571-84, 1959.
16. Ephrussi-Taylor, H. Transformations allogènes du pneumocoque, *Exp. Cell Res. 2*:589-607, 1951.
17. Alexander, H. E. and Redman, W. Transformation of type specificity of meningococci, *J. exp. Med. 97*:797-806, 1953.
18. Austrian, R. and MacLeod, C. M. Type-specific protein from pneumococcus, *J. exp. Med. 89*:439-50, 1949.
19. Austrian, R. and MacLeod, C. M. Acquisition of M protein by pneumococci through transformation reactions, *J. exp. Med. 89*:451-60, 1949.
20. Austrian, R. Observations on the transformation of pneumococcus *in vivo*, *Bull. Johns Hopk. Hosp. 91*:189-96, 1952.
21. Hotchkiss, R. D. Transfer of penicillin resistance in pneumococci by the desoxyribonucleate derived from resistant cultures, *Cold Spr. Harb. Symp. quant. Biol. 16*:457-61, 1951.